

# ACTH is a potent regulator of gene expression in human adrenal cells

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## Abstract

The adrenal glands are the primary source of mineralocorticoids, glucocorticoids, and the so-called adrenal androgens. Under physiological conditions, cortisol and adrenal androgen synthesis are controlled primarily by ACTH. Although it has been established that ACTH can stimulate steroidogenesis, the effects of ACTH on overall gene expression in human adrenal cells have not been established. In this study, we defined the effects of chronic ACTH treatment on global gene expression in primary cultures of both adult adrenal (AA) and fetal adrenal (FA) cells. Microarray analysis indicated that 48 h of ACTH treatment caused 30 AA genes and 84 FA genes to increase by greater than fourfold, with 20 genes common in both cell cultures. Among these genes were six encoding enzymes involved in steroid biosynthesis, the ACTH receptor and its accessory protein, melanocortin 2 receptor accessory protein (ACTH receptor accessory protein). Real-time quantitative PCR confirmed the eight most upregulated and one downregulated common genes between two cell types. These data provide a group of ACTH-regulated genes including many that have not been previously studied with regard to adrenal function. These genes represent candidates for regulation of adrenal differentiation and steroid hormone biosynthesis.

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## Introduction

ACTH is a 39 amino acid polypeptide predominantly synthesized in and secreted from the anterior lobe of the pituitary gland. The synthesis and secretion of ACTH are tightly controlled by the hypothalamic–pituitary–adrenal axis. Under stress conditions, the paraventricular nucleus of the hypothalamus secretes vasopressin and CRH. These two peptides regulate the anterior lobe of the pituitary gland and stimulate the secretion of ACTH. ACTH subsequently induces adrenal cortex expansion and corticosteroid production (mainly cortisol in humans). Once synthesized, cortisol in turn acts on the hypothalamus and pituitary (to suppress CRH and ACTH production) causing a negative feedback cycle. In the adrenal glands, ACTH acts by binding to specific cell surface ACTH receptors (melanocortin 2 receptor (MC2R)). MC2R is a seven membrane-spanning G-protein-coupled receptor that is primarily expressed in adrenocortical cells. Upon ligand binding, the receptor undergoes conformational changes that stimulate adenyl cyclase, leading to an increase in intracellular cAMP and subsequent activation of protein kinase A (PKA).

Although previous studies have identified some ACTH-responsive genes that are involved with the steroidogenic and growth-related effects of ACTH (Neri *et al.* 1991, Markowska *et al.* 1993, Cecim *et al.* 1996,

Gaillard *et al.* 2000, Le Roy *et al.* 2000, Simmonds *et al.* 2001, Banerjee *et al.* 2008), there is a lack of knowledge regarding the global actions of ACTH on gene expression. Given the critical role of ACTH in adrenal development, steroidogenesis, and disease, it is appropriate to further define the detailed effects of ACTH on human adrenal cell gene expression. In this study, we used oligonucleotide microarray analysis to define the effects of ACTH on gene expression in primary cultures of human adrenocortical cells. The study has defined a series of ACTH-responsive genes that greatly expands our understanding of ACTH action in humans.

## Materials and methods

### Tissue collection

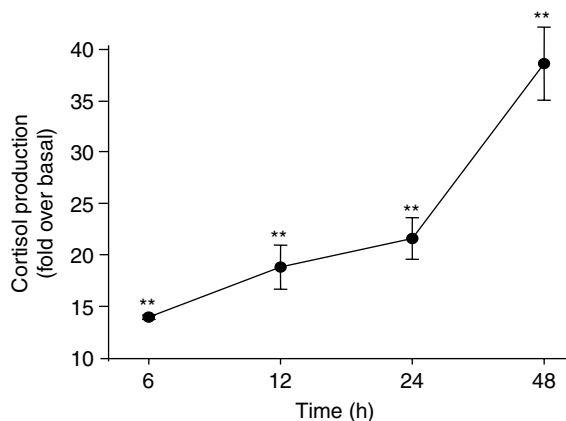
Human adult adrenal (AA) glands were obtained from cadaveric kidney donors transplanted at the Medical College of Georgia (Augusta, GA, USA) with the family's informed consent obtained by LifeLink of Georgia. Human fetal adrenal (FA) glands (14–19 weeks of gestation) were obtained from pathological examination following elective pregnancy terminations from Advanced Bioscience Resources Inc. (Alameda, CA, USA) with informed consent. The use of these tissues was approved by the Institutional Review Board

of Medical College of Georgia and the University of Alabama at Birmingham (Birmingham, AL, USA).

### Cell culture and treatment

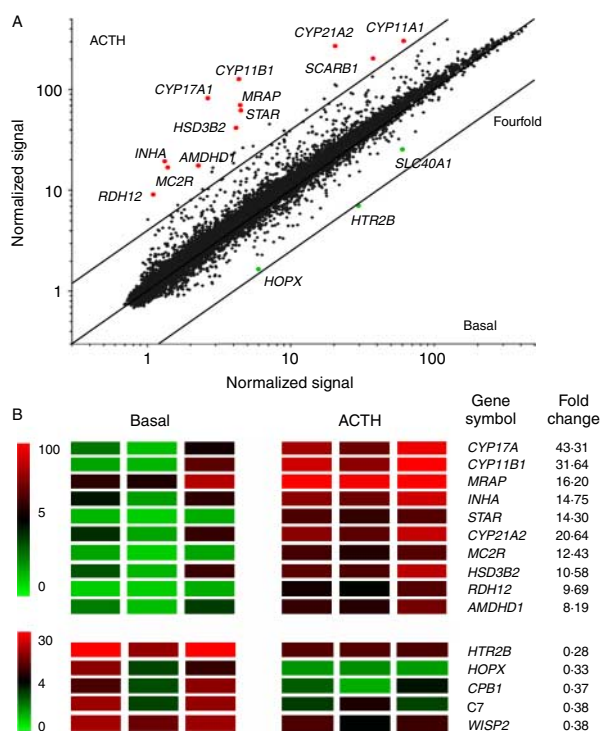
Adult adrenocortical cells were isolated with collagenase–dispase digestion as described previously (Bassett *et al.* 2004). The AAs were minced and dissociated into single cell suspension by repeated exposure of the tissue fragments to DMEM/F12 medium (Invitrogen) containing 1 mg/ml of collagenase–dispase and 0.25 mg/ml of DNase-1 (F Hoffmann-La Roche Ltd). Digestion and mechanical dispersion were carried out four times for 1 h each at 37 °C. Cells were collected between each digestion, and combined before aliquots of AA cells were frozen (2 000 000 cells per vial) and stored at –150 °C.

FAs were minced and dissociated into single cell suspensions by repeated exposure of the tissue fragments to 0.4 mg/ml collagenase (Sigma) in PBS enriched with 10% BSA (Sigma) at 37 °C for 15–30 min. After separation of the cells from the collagenase mixture by centrifugation, the cell pellets were suspended in culture medium (McCoy's 5A medium (Gibco) that contained 5% fetal bovine serum (Hyclone, Logan, UT, USA) and antibiotics/antimycotics (Gibco)). Cells were initially cultured in Falcon 75cc flasks (Becton Dickinson & Co., Lincoln Park, NJ, USA) for 2–3 days at 37 °C in a humidified atmosphere (95% air and 5% CO<sub>2</sub>) to ensure viability and lack of infection prior to experimental use.



**Figure 1** Time-dependent effects of ACTH on cortisol production in human AA cells. Primary human AA cells were prepared as described under Materials and methods, and plated at the density of 200 000 cells per well in 24-well dishes. Cells were treated with or without ACTH (10 nM) for the indicated times, and cortisol was quantified in the medium using EIA. Cortisol data were normalized to protein per well and expressed as the fold change over basal condition (untreated cells) for each time point. Results represent the mean  $\pm$  S.E.M. of data from at least three independent experiments. Three wells were analyzed for individual treatment in each experiment. Statistics were calculated using one-way ANOVA followed by Dunnett's test, comparing with baseline. \*\* $P < 0.01$ .

For experiments, AA or FA cells were subcultured and plated at a density of 200 000 cells/well in 24-well dishes and allowed to grow for 5 days in complete growth medium (10% cosmic calf serum (Hyclone) and 1% antibiotic for AA cells, and 10% cosmic calf serum, 1% ITS plus (BD Diagnostic Systems, Sparks, MD, USA), and 1% antibiotic for FA cells; Rehman *et al.* 2007). The day before treatment, the cells were changed to experimental medium (0.1% cosmic calf serum and 1% antibiotics for AA cells, and 1% cosmic calf serum, 1% ITS plus, and 1% antibiotic for FA cells) for overnight incubation followed by 48-h treatment with or without ACTH (10 nM; Organon, Bedford, OH, USA). The cells were lysed for RNA isolation, and the media were collected for steroid assays as described below.



**Figure 2** Microarray analysis of ACTH effects on AA cell gene expression. (A) Scatter plot comparing gene expression between basal and ACTH-treated AA samples. Total RNA from three sets of primary AA cell samples treated with or without ACTH was used for six oligonucleotide microarrays, and the data were combined to form this plot. The graph represents 37 846 transcripts that were found to have a signal above background level in at least one tissue sample. The transcripts with the highest variation between the basal and ACTH treatments are labeled. (B) Heatmap of the fifteen most differentially expressed transcripts between three basal and three ACTH-treated samples. The top ten upregulated and top five downregulated genes are given here. Colors represent the expression level from the median of all the samples for each probe set. Results represent data from three experiments using cells isolated from three independent donor adrenal glands. Full colour version of this figure available via <http://dx.doi.org/10.1677/JME-10-0006>.

## RNA isolation

Total RNA was extracted from human adrenal cells using the RNeasy mini kit from Qiagen following the manufacturer's protocol, and the quantity of RNA was checked spectroscopically using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Deoxyribonuclease I (Ambion Inc., Austin, TX, USA)-treated total RNA (2 µg) was reverse transcribed using the High Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA, USA) and stored at  $-80^{\circ}\text{C}$ .

## Microarray analysis

Total adrenal cell RNA from basal and ACTH-treated cells was used on genomic expression arrays performed by the Microarray Core Facility at the Medical College of Georgia. Briefly, FA RNA was hybridized to Illumina HumanRef-8 (gene targets 24 526), and AA RNA was hybridized to HumanHT-12 (gene targets 48 804) Expression BeadChips (Illumina, San Diego, CA, USA). Results were analyzed using GeneSpring GX 7.3.1 software (Silicon Genetics, Redwood City, CA, USA) and normalized to the 50th percentile of all signal values.

## Quantitative real-time RT-PCR

Inhibin, alpha (INHA), HOP homeobox (HOPX), and MC2R accessory protein (MRAP) primers were obtained from Qiagen, and the mRNA levels were detected using SYBR Green PCR master mix

(Applied Biosystems). CYP11B1, HSD3B2, CYP17, CYP21, and steroidogenic acute regulatory protein (STAR) primer/probe mix was prepared following the sequence published before (Pezzi *et al.* 2003). The Taqman gene expression assay for MC2R was purchased from Applied Biosystems. qPCRs were performed using the ABI Prism 7000 Sequence Detection System (Applied Biosystems) in a total volume of 20 µl reaction mixture following the reaction parameters recommended by the manufacturer. Relative quantification of mRNA levels between treated/untreated groups was determined using the comparative  $C_t$  value as described previously (Ye *et al.* 2007) with 18S rRNA used for normalization.

## Protein extraction and protein assay

Cells were lysed in 100 µl of Mammalian Protein Extraction Reagent (Pierce Chemical Co., Rockford, IL, USA). The protein content of samples was then determined by the bicinchoninic acid protein assay following the MicroBCA protocol (Pierce Chemical Co.).

## Steroid immunoassay

Steroid determinations were done using EIA kits for cortisol (Alpco Diagnostics, Salem, NH, USA) and dehydroepiandrosterone sulfate (DHEA-S; Diagnostic System Laboratories, Webster, TX, USA). The assays were conducted following the manufacturer recommendations, except that standard curves were prepared in the experimental cell culture medium.

**Table 1** Transcripts most regulated by ACTH treatment in adult adrenal (AA) cells. Primary human AA cell culture was treated with/without ACTH (10 nM) for 48 h, and RNA was isolated for microarray analysis. Ten genes most upregulated and five genes most downregulated by ACTH treatment in human AA are shown. The gene symbol for each transcript is given in the first column, followed by the fold change between basal and ACTH (mean fold change of three independent experiment  $\pm$  s.e.m.) treatments and *P* value. Results represent data from three experiments using cells isolated from three independent donor adrenal glands

Gene symbol	Fold change $\pm$ s.e.m.	<i>P</i> value	GenBank accession number	Gene name
<i>CYP11B1</i>	43.31 $\pm$ 19.95	0.168	NM_001026213.1	Cytochrome P450, 11 $\beta$ hydroxylase
<i>CYP17A1</i>	31.64 $\pm$ 1.12	0.001	NM_000102.3	Cytochrome P450, 17 $\alpha$ hydroxylase/17,20 lyase
<i>CYP21A2</i>	20.64 $\pm$ 9.31	0.169	NM_000500.5	Cytochrome P450, 21 hydroxylase
<i>MRAP</i>	16.20 $\pm$ 2.49	0.026	NM_178817.3	Melanocortin 2 receptor accessory protein
<i>INHA</i>	14.74 $\pm$ 0.52	0.001	NM_002191.2	Inhibin, $\alpha$
<i>STAR</i>	14.30 $\pm$ 2.36	0.030	NM_001007243.1	Steroidogenic acute regulatory protein
<i>MC2R</i>	12.42 $\pm$ 1.62	0.020	NM_000529.2	Melanocortin 2 receptor
<i>HSD3B2</i>	10.58 $\pm$ 2.54	0.064	NM_000198.2	3 $\beta$ hydroxysteroid dehydrogenase
<i>RDH12</i>	9.69 $\pm$ 4.03	0.164	NM_152443.1	Retinol dehydrogenase 12 (all-trans/9-cis/11-cis)
<i>AMDHD1</i>	8.19 $\pm$ 1.74	0.054	NM_152435.1	Amidohydrolase domain containing 1
<i>HTR2B</i>	0.28 $\pm$ 0.11	0.0221	NM_000867.3	5-Hydroxytryptamine (serotonin) receptor 2B
<i>HOPX</i>	0.33 $\pm$ 0.14	0.0431	NM_032495.4	HOP homeobox
<i>CPB1</i>	0.37 $\pm$ 0.06	0.0084	NM_001871.2	Carboxypeptidase B1
<i>C7</i>	0.38 $\pm$ 0.01	0.0001	NM_000587.2	Complement component 7
<i>WISP2</i>	0.38 $\pm$ 0.08	0.0166	NM_003881.2	WNT1-inducible signaling pathway protein 2

*P* value was calculated using paired *t*-test comparing the fold changes with/without ACTH treatment.

Results were normalized to protein per tissue culture well, and were shown as fold changes compared to basal treatment.

### Statistical analysis

Results are given as mean  $\pm$  S.E.M. Individual experiments were repeated at least three times, using cells isolated from separate adrenal glands. One-way ANOVA or paired *t*-test was performed using GraphPad Prism 3.0 (GraphPad Software, Inc., San Diego, CA, USA).

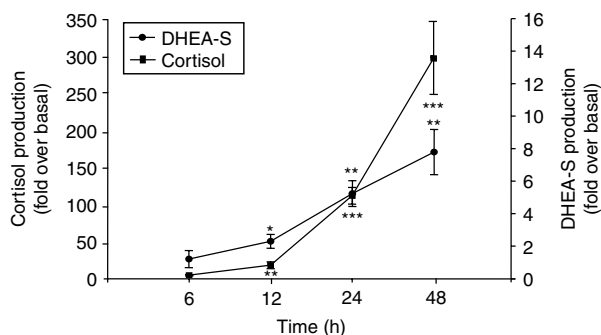
## Results

### ACTH stimulates cortisol production in AA cells

As shown in Fig. 1, treatment of AA cells with ACTH (10 nM) resulted in a significant elevation of cortisol biosynthesis. Media content of cortisol increased following ACTH treatment in a time-dependent manner. ACTH caused a significant increase in medium cortisol within 6 h, and by 48 h, ACTH increased cortisol by over 30-fold above that seen in untreated cells.

### Microarray data of AA 48-h treatment with ACTH

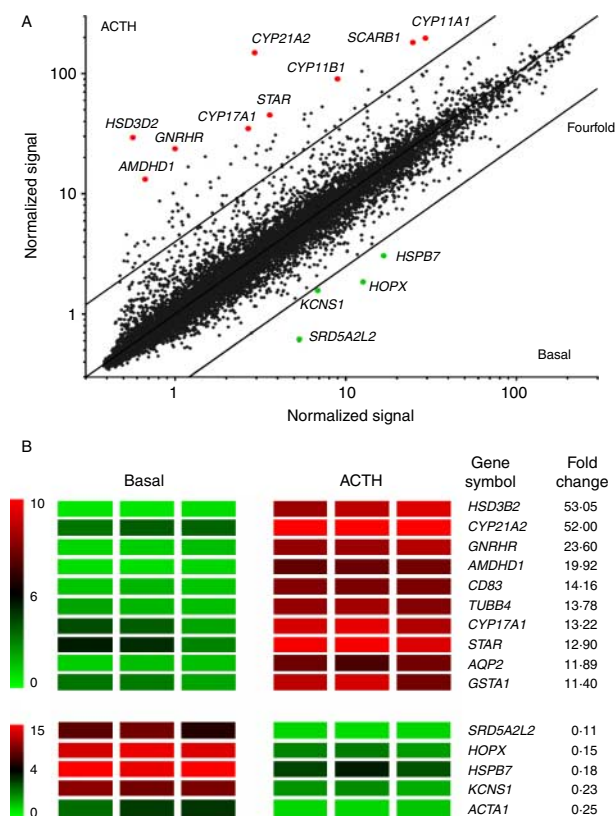
To determine the global gene changes caused by ACTH treatment in AA cells, microarray analysis was performed comparing basal and ACTH



**Figure 3** Time-dependent effects of ACTH on cortisol and DHEA-S production in FA primary cultures. Primary human FA cells were prepared as described under Materials and methods, and plated at a density of 200 000 cells per well in 24-well dishes. The day before experiments, cells were changed to 1% low serum medium overnight. Cells were treated with ACTH (10 nM) for the indicated times followed by quantification of medium cortisol and DHEA-S using EIA kits. Steroid data were normalized to protein per well and expressed as the fold change over basal conditions (untreated cells) for each time point. Results represent the mean  $\pm$  S.E.M. of data from at least three independent experiments. Three wells were analyzed for individual treatment in each experiment. Statistics were calculated using one-way ANOVA followed by Dunnett's test, comparing with baseline. \**P* < 0.05; \*\**P* < 0.01; \*\*\**P* < 0.001.

(10 nM, 48 h)-treated samples (Fig. 2A). Cells were isolated from three independent donor adrenal glands and used for independent experiments. Six microarrays were run with three sets of basal and ACTH-treated samples. Among the 37 846 genes shown in the scatter plot, 30 genes were upregulated by ACTH over fourfold, while only one gene (serotonin receptor 2B, *HTR2B*) was downregulated for more than fourfold. The top upregulated and downregulated genes are shown in the microarray heatmap (Fig. 2B) and listed in Table 1.

As shown in Fig. 2 and Table 1, all steroidogenic enzymes involved in adrenal cortisol production were



**Figure 4** Microarray analysis of ACTH effects on FA cell gene expression. (A) Scatter plot comparing gene expression between basal and ACTH-treated samples in FA cells. Total RNA from three sets of primary adrenal samples treated with or without ACTH was used for oligonucleotide microarray analysis, and the data were combined. The graph represents 18 391 transcripts that were found to have a signal above background level in at least one cell sample. The transcripts with the highest variation between basal and ACTH treatments are labeled. Results represent data from three experiments using cells isolated from three independent adrenal glands. (B) Heatmap of the 15 most differentially expressed transcripts between basal and ACTH treatments. The ten most upregulated and five most downregulated genes are shown. Colors represent the expression level from the median of all the samples for each probe set. Results represent data from three experiments using cells isolated from three independent FA glands. Full colour version of this figure available via <http://dx.doi.org/10.1677/JME-10-0006>.

increased following treatment with ACTH for 48 h. CYP17 showed the largest increase of 32-fold. This increase confirms that ACTH is a very potent steroidogenic activator that chronically increases the capacity of adrenal cells to produce steroid hormones by increasing steroidogenic enzyme expression. In addition, ACTH treatment increased the expression of its own receptor – MC2R (12-fold). Interestingly, an important component and effector of the MC2R system, the MRAP, was also upregulated by ACTH (16-fold).

### ACTH stimulates cortisol and DHEA-S production in FA

Like AA cells, human FA cells are dependent on ACTH to increase steroidogenesis. To test the effects of ACTH on human FA, we isolated cells from three separate adrenals and ran three independent experiments. As shown in Fig. 3, both cortisol and DHEA-S levels were significantly stimulated by ACTH treatment, and these effects were time-dependent, continuing to magnify with time. By 48 h, ACTH was able to increase DHEA-S by eightfold and cortisol by over 300-fold when compared to basal steroid production. Six microarrays were performed on the 48-h ACTH treatment groups (Fig. 4A), and the genes that were most upregulated and downregulated are summarized in Fig. 4B and Table 2. Among the 18 391 genes shown in the scatter plot, 84 of them were upregulated more than fourfold by 48-h ACTH treatment, and 5 genes were downregulated

for more than fourfold. Four steroidogenic enzyme genes including *HSD3B2*, *CYP21A2*, *CYP17A1*, and *STAR* were represented in the list of ten genes most affected by ACTH. In this group, the mRNA encoding *HSD3B2* increased the most at 53-fold above that seen in untreated cells.

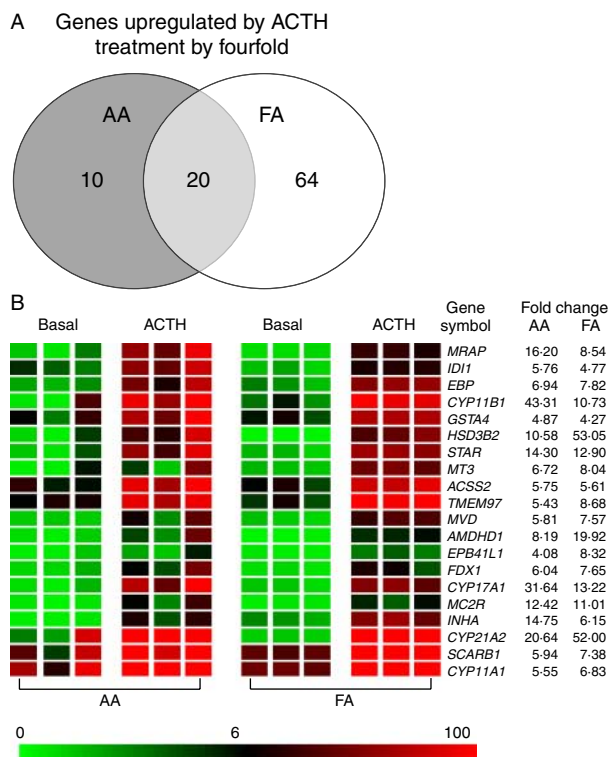
### Common genes shared by AA and FA

Comparing the pan-genomic effects of ACTH on AA and FA cells, we found a series of common genes that were increased in both cell types. When setting the threshold at a fourfold increase, there were 30 genes regulated by ACTH in AA, with 84 genes in FA (Fig. 5A). Among them, 20 genes were common, suggesting that those genes may represent a universal set of human adrenal ACTH targets and perform the critical functions associated with ACTH action. The heatmap for those 20 genes is shown in Fig. 5B, followed by the detailed fold changes in both cell types (Table 3). Among the 20 common genes, six were transcripts encoding steroidogenic enzymes/proteins, namely *STAR*, *CYP11A1*, *CYP17A1*, *HSD3B2*, *CYP21A2*, and *CYP11B1*. The ACTH receptor (MC2R) and MRAP were upregulated by ACTH in both cell cultures. There was also a significantly increased expression of the scavenger receptor type B class 1 (*SCARB1*) transcript with ACTH treatment. As the major HDL receptor in the adrenal gland, *SCARB1* is shown to be regulated by cAMP and protein kinase C (PKC) pathways. Although

**Table 2** Transcripts most regulated by ACTH treatment in fetal adrenal (FA) cells. Primary human FA cell cultures were treated with/without ACTH (10 nM) for 48 h, and RNA was isolated for microarray analysis. Ten genes most upregulated and five genes most downregulated by ACTH treatment in human FA are shown. The gene symbol of each transcript is given in the first column, followed by the fold change between basal and ACTH treatments (mean fold change of three independent experiments  $\pm$  s.e.m.) and *P* value. Result represents data from three experiments using cells isolated from three independent donor FA glands

Gene symbol	Fold change $\pm$ s.e.m.	<i>P</i> value	GenBank accession number	Gene name
<i>HSD3B2</i>	53.05 $\pm$ 4.08	0.006	NM_000198.2	3 $\beta$ hydroxysteroid dehydrogenase
<i>CYP21A2</i>	52.00 $\pm$ 4.11	0.006	NM_000500.5	Cytochrome P450, 21 hydroxylase
<i>GNRHR</i>	23.60 $\pm$ 0.67	0.001	NM_001012763.1	Gonadotropin-releasing hormone receptor
<i>AMDHD1</i>	19.92 $\pm$ 1.04	0.003	NM_152435.1	Amidohydrolase domain containing 1
<i>CD83</i>	14.16 $\pm$ 1.31	0.010	NM_004233.3	CD83 molecule
<i>TUBB4</i>	13.78 $\pm$ 1.60	0.015	NM_006087.2	Homo sapiens tubulin, $\beta$ 4
<i>CYP17A1</i>	13.22 $\pm$ 1.48	0.014	NM_000102.3	Cytochrome P450, 17 $\alpha$ hydroxylase/17,20 lyase
<i>STAR</i>	12.90 $\pm$ 1.80	0.022	NM_001007243.1	Steroidogenic acute regulatory protein
<i>AQP2</i>	11.89 $\pm$ 5.60	0.191	NM_000486.3	Aquaporin 2 (collecting duct)
<i>GSTA1</i>	11.40 $\pm$ 1.85	0.030	NM_145740.2	Glutathione S-transferase A1
<i>SRD5A2L2</i>	0.11 $\pm$ 0.02	0.0010	NM_001010874.3	Steroid 5 alpha-reductase 2-like 2
<i>HOPX</i>	0.15 $\pm$ 0.01	0.0019	NM_032495.4	HOP homeobox
<i>HSPB7</i>	0.18 $\pm$ 0.04	0.0002	NM_014424.3	Heat shock 27 kDa protein family, member 7 (cardiovascular)
<i>KCNK1</i>	0.23 $\pm$ 0.02	0.0004	NM_002251.3	Potassium voltage-gated channel, delayed-rectifier, subfamily S, member 1
<i>ACTA1</i>	0.25 $\pm$ 0.02	0.0010	NM_001100.3	Skeletal muscle $\alpha$ -actin

*P* value was calculated using paired *t*-test comparing the fold changes after ACTH treatment.



**Figure 5** Common genes that are increased by ACTH treatment in AA and FA cells. (A) Venn diagram of genes upregulated by ACTH by at least fourfold in AA and FA cells. Grey, AA cell; white, FA cell. (B) Heatmap of the 20 ACTH upregulated genes in both AA and FA cells. Colors represent the expression level from the median of all the samples for each probe set. Results represent data from three experiments using cells isolated from three independent donor adrenal glands. Full colour version of this figure available via <http://dx.doi.org/10.1677/JME-10-0006>.

it is established that SCARB1-mediated selective uptake is not the major course of cholesterol supply in the human adrenal, this change of SCARB1 expression may suggest that HDL plays other important roles in adrenal function. Real-time qPCR was used to confirm microarray data for the eight genes that were most upregulated by ACTH and the one gene that was downregulated in both FA and AA cell types (Fig. 6). All nine genes examined by qPCR were regulated in a similar manner to that seen by microarray, thus confirming that they are ACTH-responsive genes in AA and FA cells.

## Discussion

ACTH is the major regulator of adrenal steroidogenesis, and has been shown to increase aldosterone, cortisol, and DHEA production. Aside from steroidogenic enzymes, several other gene targets of ACTH have been identified. Schimmer *et al.* (2006) identified

a series of ACTH-responsive genes using microarray analysis in the Y-1 mouse adrenal tumor cell model. However, there have been no studies about the global actions of ACTH on human adrenal cell gene expression. Herein, we used primary cultures of human adrenocortical cells as models and describe the genomic effects of ACTH.

The steroidogenic action of ACTH in the adrenal cortex has been well studied, and is believed to be mediated by cAMP and PKA via two temporal responses (Cote & Yasumura 1975, Durand *et al.* 1987, Simpson *et al.* 1987, Sewer & Waterman 2003). The acute response is mediated by the activity of the STAR protein, which leads to mobilization of cholesterol from cellular stores to the inner mitochondrial membrane where cholesterol side-chain cleavage (CYP11A1) is located (Fleury *et al.* 1996, 1998, Ariyoshi *et al.* 1998, Clark & Combs 1999, Lehoux *et al.* 2003). Studies in fetal and AAs across several species have shown that the chronic response of ACTH includes the activation of genes encoding the steroidogenic enzymes, HSD3B2, CYP21, CYP17, and CYP11B1 (Di Blasio *et al.* 1990, Tangalakis *et al.* 1990, Neri *et al.* 1991, Mason *et al.* 1993, Lebrethon *et al.* 1994, Galtier *et al.* 1996, Coulter & Jaffe 1998, Simmonds *et al.* 2001, Bassett *et al.* 2004, Ismat *et al.* 2005, Su *et al.* 2005, Carey *et al.* 2006, Schimmer *et al.* 2006, Banerjee *et al.* 2008, Fernandez-Ranvier *et al.* 2008). These gene targets were confirmed in the current study in AA and FA cells using microarray analysis. All the steroidogenic enzymes needed for cortisol synthesis were upregulated by ACTH treatment.

In agreement with previous studies (Mountjoy *et al.* 1994, Le Roy *et al.* 2000, Su *et al.* 2005, Carey *et al.* 2006, Johnston *et al.* 2007, Rehman *et al.* 2007), we found that the ACTH receptor MC2R was significantly stimulated by ACTH incubation (12-fold). We also noticed a significant increase in MRAP mRNA levels. As an essential component for MC2R function, MRAP was first identified by Metherell *et al.* (2004). Its mutation has been shown to be responsible for some cases of familial glucocorticoid deficiency type 2, in which there is elevated ACTH, but circulating cortisol is low or absent (Metherell *et al.* 2005, Rumie *et al.* 2007). By stimulating expression of both MC2R and MRAP concomitantly, ACTH forms a feed forward loop leading to amplification of responsiveness.

As mentioned above, the only other microarray study regarding ACTH genomic effects on adrenal function was performed using the Y-1 mouse adrenal tumor cells (Schimmer *et al.* 2006). In this study, Y-1 adrenal cells were treated with ACTH (20 nM) for 24 h, and 588 genes were found to be significantly increased. Compared to their results, we also confirmed upregulation of steroidogenic enzymes, as well as two other transcripts, SCARB1 and INHA. When treating Y-1

**Table 3** Genes with increased expression in both human adult adrenal (AA) and fetal adrenal (FA) cells. The genes upregulated more than fourfold in AA and FA cells after 48-h ACTH treatment were compared, and common genes are listed below. The gene symbol and full name for each transcript are given in the first and last columns, followed by the fold change between basal and ACTH treatments and gene accession number

Gene symbol	Fold change in HAA	Fold change in HFA	GenBank accession number	Gene name
<i>CYP11B1</i>	43.31	10.73	NM_001026213.1	Cytochrome P450, 11 $\beta$ hydroxylase
<i>CYP17A1</i>	31.64	13.22	NM_000102.3	Cytochrome P450, 17 $\alpha$ hydroxylase/17,20 lyase
<i>CYP21A2</i>	20.64	52.00	NM_000500.5	Cytochrome P450, 21 hydroxylase
<i>MRAP</i>	16.20	8.54	NM_178817.3	Melanocortin 2 receptor accessory protein (MRAP)
<i>INHA</i>	14.75	6.15	NM_002191.2	Inhibin, $\alpha$
<i>STAR</i>	14.30	12.90	NM_001007243.1	Steroidogenic acute regulatory protein
<i>MC2R</i>	12.42	11.01	NM_000529.2	Melanocortin 2 receptor
<i>HSD3B2</i>	10.58	53.05	NM_000198.2	3 $\beta$ hydroxysteroid dehydrogenase
<i>AMDHD1</i>	8.19	19.92	NM_152435.1	Amidohydrolase domain containing 1
<i>EBP</i>	6.94	7.82	NM_006579.1	Emopamil binding protein (sterol isomerase)
<i>MT3</i>	6.72	8.04	NM_005954.2	Metallothionein 3
<i>FDX1</i>	6.04	7.65	NM_004109.3	Ferredoxin 1
<i>SCARB1</i>	5.94	7.38	NM_005505.3	Scavenger receptor class B, type 1
<i>MVD</i>	5.81	7.57	NM_002461.1	Mevalonate (diphospho) decarboxylase
<i>IDI1</i>	5.76	4.77	NM_004508.2	Isopentenyl-diphosphate delta isomerase 1
<i>ACSS2</i>	5.75	5.61	NM_018677.2	Acyl-CoA synthetase short-chain family member 2
<i>CYP11A1</i>	5.55	6.83	NM_000781.2	Cytochrome P450, side-chain cleavage
<i>TMEM97</i>	5.43	8.68	NM_014573.2	Transmembrane protein 97
<i>GSTA4</i>	4.87	4.27	NM_001512.2	Glutathione S-transferase, A4
<i>EPB41L1</i>	4.08	8.32	NM_177996.1	Erythrocyte membrane protein band 4.1-like 1

mouse adrenal cells with ACTH, Schimmer *et al.* (2006) was able to detect a fourfold change in SCARB1 transcript, suggesting this multifunction HDL receptor is a common ACTH target shared between humans and mice. INHA was also stimulated in FA cells and shown to increase in Y-1 cells (Schimmer *et al.* 2006). Although normally associated with gonadal function, INHA has been shown to influence adrenal activity. INHA expression appears to correlate with CYP17 expression in human and rat adrenocortical tissues (Wang *et al.* 2003, Hofland *et al.* 2006), and can antagonize the inhibitory effects of activin and bone morphogenetic protein on *Cyp17* mRNA in a mouse adrenocortical cell line (Farnworth *et al.* 2006).

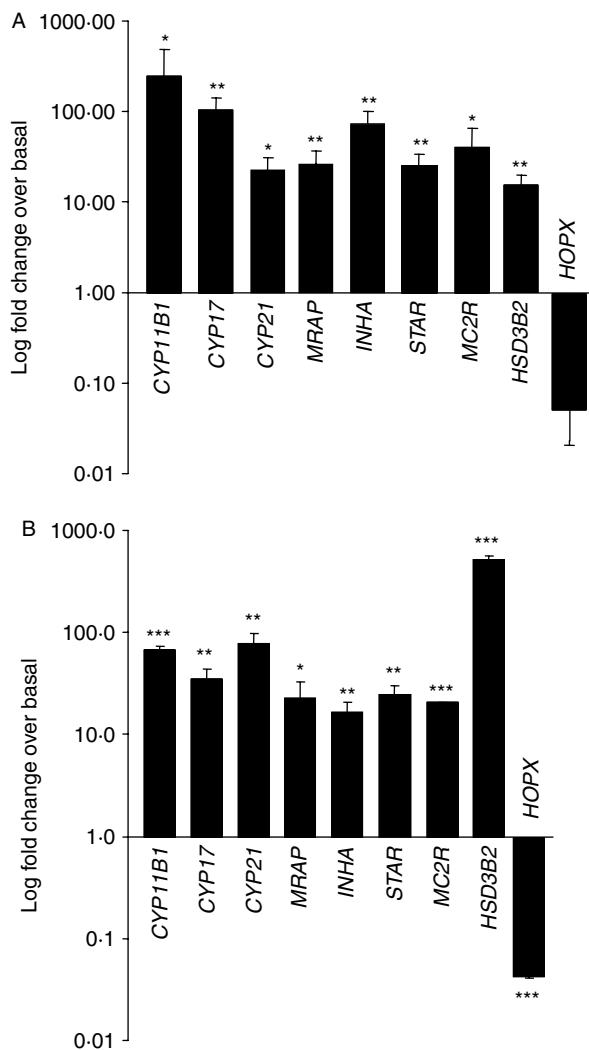
In addition to the genes previously mentioned, *RDH12* was also among the top genes upregulated by ACTH in AA cells. *RDH12* is an efficient NADPH-dependent retinal reductase, displaying high activity toward 9-*cis*- and all-*trans*-retinol (Haeseleer *et al.* 2002, Janecke *et al.* 2004, Maeda *et al.* 2006). *RDH12* overexpression in HEK293 cells was able to convert dihydrotestosterone to androstanediol (Keller & Adamski 2007); however, its ability to produce androgens has not been validated in adrenocortical cells. The functions of these candidates in ACTH regulation of the adrenal gland need further elucidation.

Of all the genes upregulated in human FA, one interesting finding is the *GNRHR* gene. In the previous

study, we demonstrated that *GNRHR* is the most differentially expressed G-protein-coupled receptor between AA and FA (Xing *et al.* 2009). In the current study, *GNRHR* transcript was also greatly stimulated in FA after ACTH treatment, suggesting its regulation by the ACTH, cAMP, and PKA pathways. The role of *GNRHR* in the human FA, however, remains to be determined.

The comparison of AA and FA microarray data gave 20 common genes that were upregulated by ACTH in both cell models. Comparison of these unique models provided a series of broad ACTH target genes. Other published targets of ACTH in adrenal glands include a series of transcription factors (Sewer & Waterman 2003), bTREK-1 potassium channel (Liu *et al.* 2008), and genes involved in cell proliferation (Lotfi *et al.* 2000, Rocha *et al.* 2003). However, due to the chronic treatment period (48 h) used in the current study, we were not able to detect increases of these rapid response genes in our microarray analysis results.

Among genes that ACTH decreased expression by greater than fourfold, *HOPX* is the only one seen in both AA and FA cells. *HOPX* was first identified in lung cancer as a potential tumor repressor gene (Chen *et al.* 2003, 2007). Recently, *HOPX* was shown to be involved in cardiac development (Chen *et al.* 2002, Ismat *et al.* 2005), and function by physically interacting with serum response factor (SRF) and the inhibition of



**Figure 6** Analysis of common ACTH-responsive gene expression in AA and FA cells using qPCR. Primary AA (A) and FA (B) cells were incubated with or without ACTH for 48 h. Transcript levels of the eight most upregulated and one downregulated genes, selected from those found in Table 3 and Fig. 5, were quantified using qPCR. Data were normalized to 18S rRNA and expressed as fold change over the basal treatment. Results represent data from three experiments using cells isolated from three independent adrenal glands. Statistics were calculated using paired Student's *t*-test, comparing the normalized  $C_t$  values of basal versus ACTH treatment. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .

SRF-dependent transcription (Yamaguchi *et al.* 2009). Although no studies have examined HOPX function in the adrenal gland, its tumor repressor activity and the downregulation by ACTH treatment suggest that it may be relevant to adrenal proliferation.

The WNT1-inducible signaling pathway protein 2 (WISP2) was also significantly downregulated after ACTH treatment in AA cells. WISP2 is a member of the connective tissue growth factor/cysteine-rich 61/nephroblastoma overexpressed family, and is

a potential target for WNT1 regulation in the mouse model (Pennica *et al.* 1998). Increased expression of WISP is detected in human colon cancer and several breast cancer cell lines (Pennica *et al.* 1998, Dhar *et al.* 2007, 2008, Banerjee *et al.* 2008). Studies by Stratakis *et al.* have suggested the involvement of this gene in adrenal disease, specifically primary pigmented nodular adrenocortical disease (Bourdeau *et al.* 2004, Horvath *et al.* 2006, Iliopoulos *et al.* 2009). However, in our system, we found that the PKA pathway represses the expression of WISP2 gene. The other ACTH-repressed gene in AA cells was *HTR2B*. Initially characterized in isolated rat stomach fundus (Clineschmidt *et al.* 1985, Komada & Yano 2007), *HTR2B* is a widely expressed G-protein-coupled receptor, functioning through PKC-dependent ERK phosphorylation (Cox & Cohen 1995, 1996, Li *et al.* 2008, Wouters *et al.* 2009). A study comparing benign adrenocortical tumors and adrenocortical carcinoma identified *HTR2B* as a potential diagnosis biomarker for distinguishing benign from malignant adrenocortical tumors (Fernandez-Ranvier *et al.* 2008). This suggested that *HTR2B* may be involved in adrenal cell growth regulation.

In summary, by applying a microarray approach, we defined the genomic effects of ACTH in human adult and FA primary cultures. The newly defined adrenal ACTH-responsive genes can provide clues to the mechanism of ACTH-regulated steroidogenesis and cell growth, and may lead to further understanding of the global functions of ACTH in the adrenal gland. This knowledge may be helpful in solving and developing treatments for adrenal diseases including hyperplasia and tumors.

## Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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